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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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[Continued on next page]

(54) Title: NOVEL CELL WALL ANCHOR PROTEINS DERIVED FROM YEAST, GENES THEREOF AND CELL SURFACE EXPRESSION SYSTEMS USING THE SAME

(57) Abstract: The present invention provides novel cell wall anchor proteins derived from yeast, genes thereof and the genetic method for tethering polypeptide to the yeast cell wall using the same. Particularly, the present invention provides the novel GPI(glycosyl phosphatidyl inositol)-anchor protein genes, *SED1, GAS1, TIP1* and *CWP1*, and their proteins, *PIR2* cell wall protein gene and its protein derived from *Hansenula polymorpha*, and the cell surface expression system using them which immobilize foreign enzymes or polypeptides on the cell wall of a microbial cell. In addition, the present invention provides the cell surface expression system using *WSC1* gene and it s protein derived from yeasts, including *Hansenula polymorpha* and *Saccharomyces cerevisiae*, and *STA1* gene and its protein derived from *Saccharomyces diastaticus*. The cell surface expression system of the present invention expects an immobilization effect as biocatalysts by adhereing a desired protein to the cell surface, and provides a means of altering target protein characteristics such as binding affinity and stability by library screening.

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patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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Published:
— *with international search report*
— *with amended claims*

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : CHOI, Eui-Sung
Dasol Apt. 102-507, #395-3, Kung-dong, Yusong-ku, Taejon 305-335,
Republic of Korea/

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ***Escherichia coli*** **DH5@/HpPIR2**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 0827BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **July 11 2000.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): BAE, Kyung Sook, Director Date: **July 15 2000**

Form BP/4 (KCTC Form 17)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : CHOI, Eui-Sung
Dasol Apt. 102-507, #395-3, Kung-dong, Yusong-ku, Taejon 305-335, Republic of Korea/

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli **DH5@/HpGAS1**	**KCTC 0828BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

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[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **July 11 2000.**

IV. RECEIPT OF REQUEST FOR CONVERSION

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Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	BAE, Kyung Sook, Director Date: **July 15 2000**

Form BP/4 (KCTC Form 17) Sole Page

What is Claimed is

1. A novel cell wall protein gene *HpSED1* and its homologues represented by the SEQ. ID. NO: 1, derived from *Hansenula polymorpha*.

2. An *E. coli* transformant (Accession No. KCTC 0825BP) transformed with a recombinant vector containing 4 kb *EcoRI* fragment of *HpSED1* gene of claim 1.

3. A novel cell wall protein gene *HpPIR2* and its homologues represented by the SEQ. ID. NO: 4, derived from *Hansenula polymorpha*.

4. An *E. coli* transformant (Accession No. KCTC 0827BP) transformed with a recombinant vector containing 5.5 kb *SalI* fragment of *HpPIR2* gene of claim 3.

5. A novel cell wall protein gene *HpGAS1* and its homologues represented by the SEQ. ID. NO: 5, derived from *Hansenula polymorpha*.

6. An *E. coli* transformant (Accession No. KCTC 0828BP) transformed with a recombinant vector

containing 3 kb *Pst*I fragment of *HpGAS1* gene of claim 5.

7. A novel cell wall protein gene *HpTIP1* and its homologues represented by the SEQ. ID. NO: 6, derived from *Hansenula polymorpha*.

8. An *E. coli* transformant (Accession No. KCTC 0824BP) transformed with a recombinant vector containing 3.5 kb *Xba*I fragment of *HpTIP1* gene of claim 7.

9. A novel cell wall protein gene *HpCWP1* and its homologues represented by the SEQ. ID. NO: 7, derived from *Hansenula polymorpha*.

10. An *E. coli* transformant (Accession No. KCTC 0826BP) transformed with a recombinant vector containing 6 kb *Sal*I fragment of *HpCWP1* gene of claim 9.

11. A surface expression system for expressing foreign polypeptides or proteins on the surface of a cell, wherein a polypeptide or its partial fragment, encoded by the DNA sequence of claim 1, 3, 5, 7 and 9, its homologues or its partial fragments, is used as an

expression mediator which localizes the foreign polypeptides or proteins onto the cell surface.

12. A surface expression system for expressing foreign polypeptides or proteins on the surface of a cell, wherein a polypeptide or its partial fragment, encoded by an isolated DNA derived from *Saccharomyces diastaticus*, containing the base sequence *STA1* represented by the SEQ. ID. NO: 9 or its partial fragment, is used as an expression mediator which localizes the foreign polypeptides or proteins onto the cell surface.

13. A surface expression system for expressing foreign polypeptides or proteins on the surface of a cell, wherein a polypeptide or its partial fragment encoded by *HpWSC1* derived from *H. polymorpha*, containing the base sequence represented by the SEQ. ID. NO:8, or a polypeptide or its partial fragment encoded by the base sequence *WSC1* derived from *S. cerevisiae*, is used as an expression mediator which localizes the foreign polypeptides or proteins onto the cell surface.

14. The surface expression system as set forth in any of claims 11 to 13 , wherein the cell is a species

selected from the group consisting of yeast spp., including *Candida* spp., *Debaryomyces* spp., *Hansenula* spp., *Kluyveromyces* spp., *Pichia* spp., *Schizosaccharomyces* spp., *Yarrowia* spp., *Saccharomyces* spp.; and *mold* spp., including *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp.

AMENDED CLAIMS

[received by the International Bureau on 26 November 2001 (26.11.01); original claims 1-14 replaced by amended claims 1-19 (4 pages)]

1. A novel cell wall protein gene *HpSED1* and its homologues represented by the SEQ. ID. NO: 1, derived from *Hansenula polymorpha*.

2. (added) A novel cell wall protein HpSed1p and its homologues represented by the SEQ. ID. NO: 11, encoded by the *HpSED1* gene of claim 1.

3. An *E. coli* transformant (Accession No. KCTC 0825BP) transformed with a recombinant vector containing 4 kb *EcoRI* fragment of *HpSED1* gene of claim 1.

4. A novel cell wall protein gene *HpPIR2* and its homologues represented by the SEQ. ID. NO: 4, derived from *Hansenula polymorpha*.

5. (added) A novel cell wall protein HpPir2p and its homologues represented by the SEQ. ID. NO: 12, encoded by the *HpPIR2* gene of claim 4.

6. An *E. coli* transformant (Accession No. KCTC 0827BP) transformed with a recombinant vector containing 5.5 kb *Sal*I fragment of *HpPIR2* gene of

7. A novel cell wall protein gene *HpGAS1* and its homologues represented by the SEQ. ID. NO: 5, derived from *Hansenula polymorpha*.

8. (Added) A novel cell wall protein HpGas1p and its homologues represented by the SEQ. ID. NO: 14, encoded by the *HpGAS1* gene of claim 7.

9. An *E. coli* transformant (Accession No. KCTC 0828BP) transformed with a recombinant vector containing 3 kb *Pst*I fragment of *HpGAS1* gene of claim 7.

10. A novel cell wall protein gene *HpTIP1* and its homologues represented by the SEQ. ID. NO: 6, derived from *Hansenula polymorpha*.

11. (added) A novel cell wall protein HpTip1p and its homologues represented by the SEQ. ID. NO: 13, encoded by the *HpTIP1* gene of claim 10.

12. An *E. coli* transformant (Accession No. KCTC 0824BP) transformed with a recombinant vector containing 3.5 kb *Xba*I fragment of *HpTIP1* gene of

13. A novel cell wall protein gene *HpCWP1* and its homologues represented by the SEQ. ID. NO: 7, derived from *Hansenula polymorpha*.

14. (added) A novel cell wall protein HpCwp1p and its homologues represented by the SEQ. ID. NO: 15, encoded by the *HpCWP1* gene of claim 13.

15. An *E. coli* transformant (Accession No. KCTC 0826BP) transformed with a recombinant vector containing 6 kb *Sal*I fragment of *HpCWP1* gene of claim 13.

16. A surface expression system for expressing foreign polypeptides or proteins on the surface of a cell, wherein a polypeptide or its partial fragment, encoded by the DNA sequence of claim 1, 4, 7, 10 and 13, its homologues or its partial fragments, is used as an expression mediator which localizes the foreign polypeptides or proteins onto the cell surface.

17. A surface expression system for expressing foreign polypeptides or proteins on the surface of a cell, wherein a polypeptide or its partial fragment,

encoded by an isolated DNA derived from *Saccharomyces diastaticus*, containing the base sequence *STA1* represented by the SEQ. ID. NO: 9 or its partial fragment, is used as an expression mediator which localizes the foreign polypeptides or proteins onto the cell surface.

18. A surface expression system for expressing foreign polypeptides or proteins on the surface of a cell, wherein a polypeptide or its partial fragment encoded by *HpWSC1* derived from *H. polymorpha*, containing the base sequence represented by the SEQ. ID. NO:8, or a polypeptide or its partial fragment encoded by the base sequence *WSC1* derived from *S. cerevisiae*, is used as an expression mediator which localizes the foreign polypeptides or proteins onto the cell surface.

19. The surface expression system as set forth in any of claims 16 to 18, wherein the cell is a species selected from the group consisting of yeast spp., including *Candida* spp., *Debaryomyces* spp., *Hansenula* spp., *Kluyveromyces* spp., *Pichia* spp., *Schizosaccharomyces* spp., *Yarrowia* spp., *Saccharomyces* spp.; and *mold* spp., including *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp.

FIG. 1

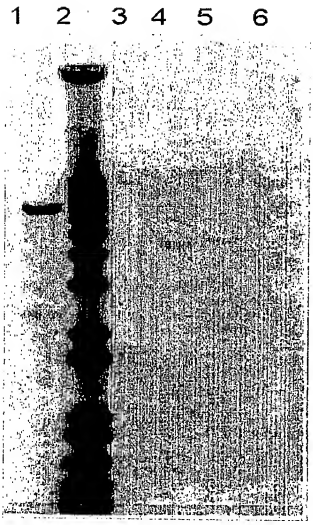


FIG. 2a

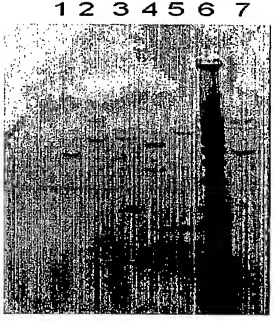


FIG. 2b

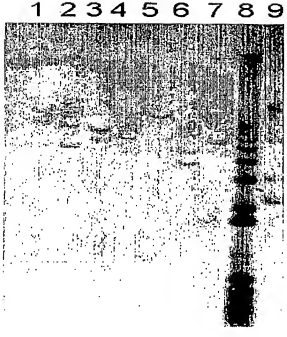
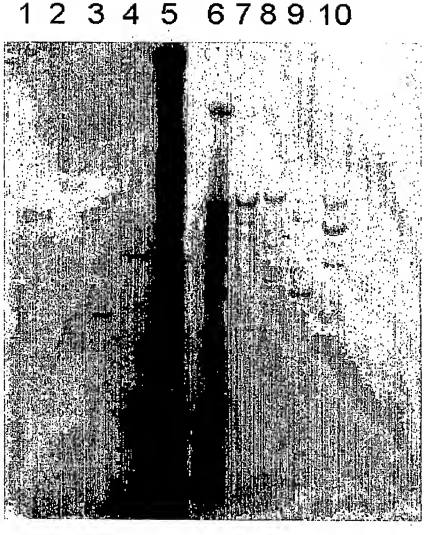


FIG. 3



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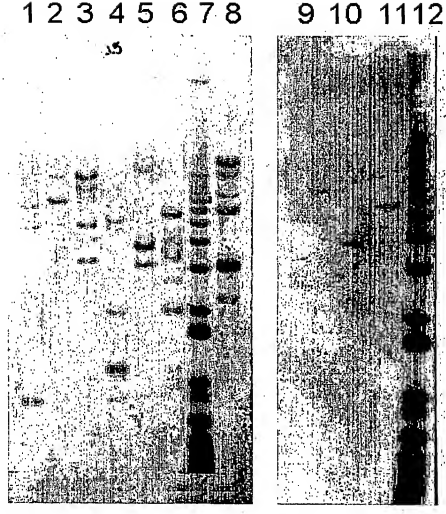
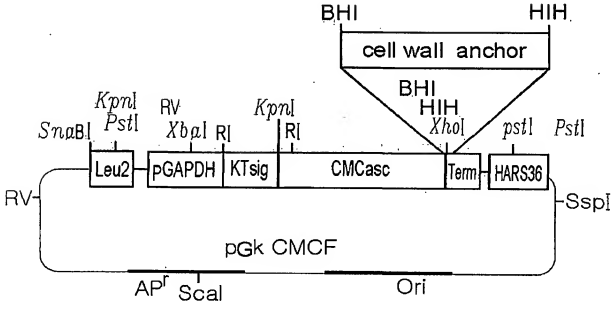


FIG. 5

FIG. 6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HIH
cell wall anchor
BHI
HIH
KpnI
PstI
SnaBI
RV
XbaI
RI
KpnI
RI
XhoI
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PstI
Leu2
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Term
HARS36
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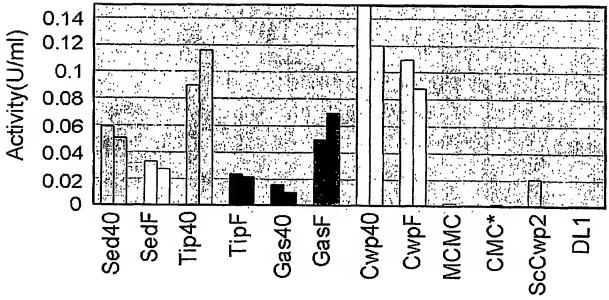
SspI
pGk CMCF
AP^r
ScaI
Ori

FIG. 7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0.14
0.12
0.1
0.08
0.06
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0.02
0
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GasF
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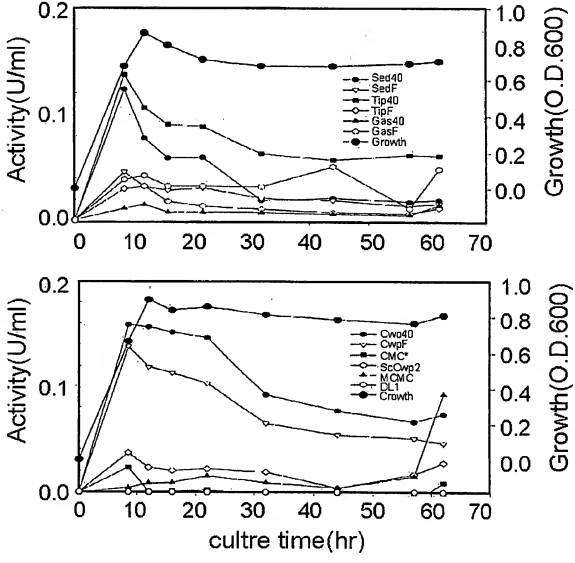
CwpF
MCMC
CMC*
ScCwp2
DL1

FIG. 8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TipF
Gas40
GasF
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ScCwp2
MCMC
DL1
Crowth
cultre time(hr)

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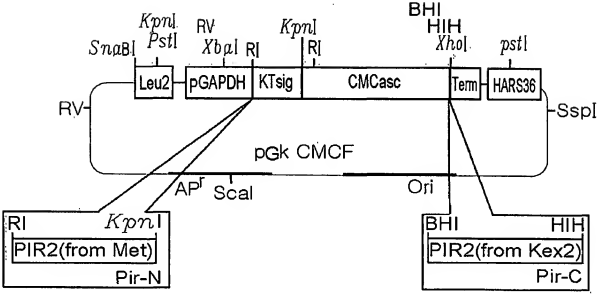
FIG. 9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XbaI
RI
KpnI
RI
BHI
HIH
XhoI
pstI
Leu2
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KTsig
CMCasc
Term
HARS36
RV
SspI
pGk CMCF
APr
ScaI
Ori
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PIR2(from Met)
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BHI
HIH
PIR2(from Kex2)
Pir-C

FIG. 10a

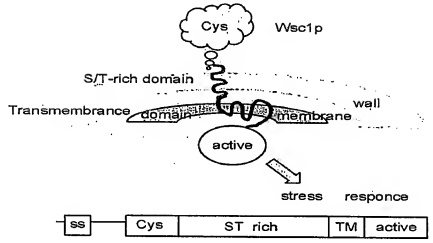


FIG. 10b

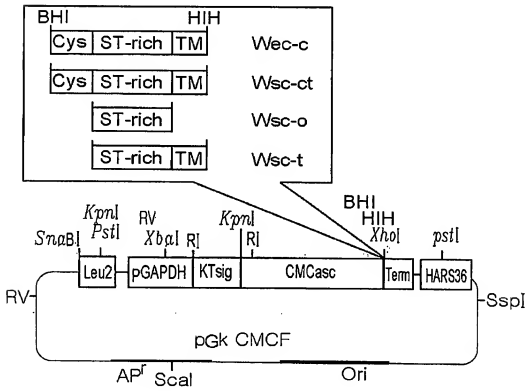
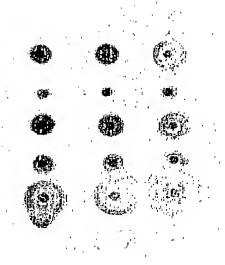


FIG. 11



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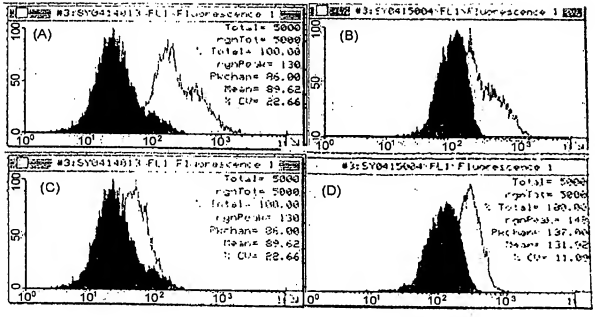

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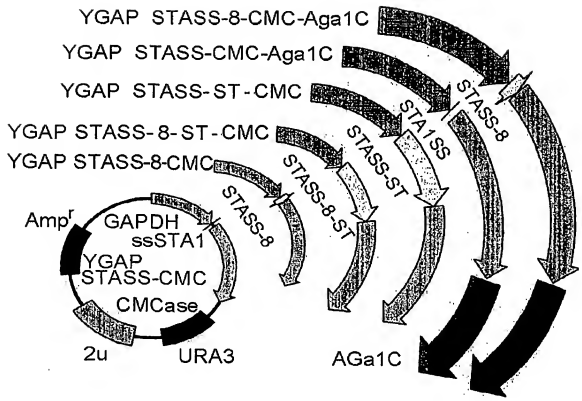
FIG. 13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STASS-8-ST
STASS-8
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FIG. 14

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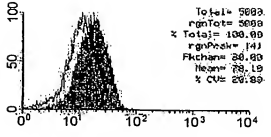
FIG. 15



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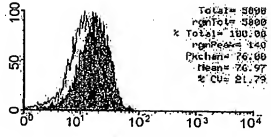

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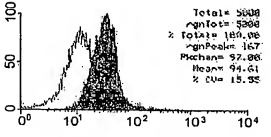

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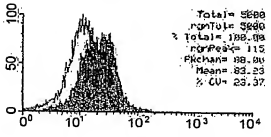

L3262 SS-TS-CMC

SEQUENCE LISTING

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<120> Novel cell wall anchor proteins derived from yeast, genes thereof and cell surface expression systems using the same

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Ser Ile Val Thr Asn Gly Lys Thr Tyr Thr Val Thr Gly Ala Thr Thr
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Leu Thr Ile Thr Asp Cys Pro Cys Thr Lys Lys Lys Val Ile Thr Thr
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Val Glu Lys Arg Gln Val Val Ser Gln Ile Gly Asp Gly Gln Ile Gln
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Ala Thr Thr Asn Thr Glu Lys Glu Thr Ser Lys Ser Ser Thr Ser Thr
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Ala Val Val Thr Gln Ile Gly Asp Gly Gln Ile Gln Ala Thr Thr Ser
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Asp Val Val Ser Gln Ile Thr Asp Gly Gln Ile Gln Ala Thr Thr Ser
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Thr Lys Ala Ser Ser Ala Thr Thr Ser Gly Val Ile Ser Gln Ile Ser
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Asp Gly Gln Ile Gln Ala Ser Ser Thr Ala Ser Ser Lys Thr Ser Thr
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Ala Ser Ser Ser Thr Ala Thr Gly Asp Tyr Val Thr Ser Val Ser Cys
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Lys Lys Glu Gly Ala Leu Ala Met Thr Leu Lys Asp Gly Ile Leu Tyr
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Gly Tyr Ala Ile Leu Ala Ala Arg Gln Cys Ser Pro Thr Gly Ser Glu
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Met

NOVEL CELL WALL ANCHOR PROTEINS DERIVED FROM YEAST, GENES THEREOF AND CELL SURFACE EXPRESSION SYSTEMS USING THE SAME

FIELD OF THE INVENTION

The present invention relates to novel cell wall proteins derived from yeast, genes thereof, and surface expression systems using the same. More particularly, the present invention relates to surface expression systems using four *Hansenula polymorpha*-derived, GPI (glycosylphosphatidylinositol)-anchor proteins, HpSed1p, HpGas1p, HpTip1p and HpCwp1p, and a *Hansenula polymorpha*-derived cell wall protein, HpPir2p for the expression of foreign proteins onto the cell surface of *Hansenula polymorpha* and other yeasts. Also, the present invention relates to surface expression systems using proteins coded by *Hansenula polymorpha*-derived *HpWSC1* and *Saccharomyces diastaticus*-derived *STA1* for the expression of foreign proteins on the cell surface of *Hansenula polymorpha* and other yeasts.

BACKGROUND ART OF THE INVENTION

In recent years, active research have been

directed to expression of desired foreign proteins on the surface of single cell organisms, such as bacteriophages, bacteria, and yeasts, because proteins expressed on the cell surface have various useful applications, including production of novel vaccines, screening of various antigens and antibodies, immobilization of useful enzymes onto the cell surface, and the like.

At first, the expression of foreign proteins on the cell surface was applied to the screening of epitopes and antigenicity-determining peptide fragments, with the aim of stable vaccine production. Before then, the production of vaccine had been achieved by the selection of the mutants, which showed stable and continuous titers from the randomly mutated pathogen library. However, the vaccines produced in the conventional manner are likely to lose their antigenicity when being administered via oral routes to humans or animals. Much effort has been made to overcome these problems using the live oral vaccines displayed on the cell surface.

In one strategy to express antigenic proteins on the cell surface, endogenous cell surface proteins are used to guide these proteins onto the cell surface. For example, a gene encoding a cell surface protein is fused to a gene encoding an antigenic protein, and the

resulting recombinant gene is introduced into Gram-negative bacteria to express the fusion protein on the cell surface. The antigenic protein passenger in the fusion protein vehicle can act as an effective antigen to elicit immune responses. Especially, Gram-negative bacteria are very efficient for this purpose, because the lipopolysaccharide (LPS) presenting on their cell envelope enhances the antigenicity of the fusion protein expressed on the cell surface.

As a rule, proteins to be secreted or expressed on cell surfaces have secretion signals, which allow the nascent proteins to pass through the cytoplasmic membrane, in their primary sequence. To be expressed on the cell surface of Gram-negative bacteria, a protein translocates across the cytoplasmic membrane and the periplasmic space and embedded in the outer membrane so as to protrude to the outer membrane. In bacteria, several enzymes and toxins have secretion signals and (or) targeting signals, which induce the proteins to target regions. Therefore, using such a secretion signal or targeting signal with the suitable promoter can successfully perform localization of a foreign protein on cell surfaces.

Thus far, extensive attempts have been made to utilize surface proteins of Gram-negative bacteria in expressing foreign proteins of interest onto the cell

surface. The surface proteins used for the localization of foreign proteins can be largely classified into outer membrane proteins, lipoproteins, secretory proteins, and cell surface organelle proteins.

LamB, PhoE and OmpA, known as outer membrane proteins of Gram-negative bacteria, have been used for the production of foreign proteins on cell surfaces. When the outer membrane proteins are employed, foreign proteins are limited in size, because they must be inserted into the loops protruded out of the cell surface. Additionally, since the C- and N-termini of the foreign proteins to be inserted are required to be located near each other in the three dimensional structure, both termini must be brought close to each other by the use of a peptide linkage when the distance therebetween is large.

In fact, where LamB or PhoE is used, insertion of a foreign polypeptide as large as or larger than 50-60 amino acids failed to construct a stable membrane protein by the steric hindrance. (Charbit et al., *J. Immunol.*, 1997, 139, 1658-1664; Agterberg et al., *Vaccine*, 1990, 8, 85-91). To solve this problem, an OmpA fragment containing a minimum target signal necessary for proper location was tried. For example, β-lactamase linked to the C-terminus of a target

signal of OmpA was successfully expressed on the cell surface. Protein translocation from the cytoplasm to the outer membrane was achieved by the fusion of the signal sequence for the lipoprotein Lpp of *E. coli* to the N-terminus of OmpA (Francisco et al., *Proc. Natl. Acad. Sci. USA*, 1992, 489, 2713-2717).

As described above, use of bacterial outer membrane proteins in cell surface display of foreign proteins requires linkage between the foreign proteins and appropriate outer membrane proteins at a gene level, so as to synthesize fusion proteins capable of passing through the cytoplasmic membrane and being stably embedded in the outer membrane. Suitable surface anchoring motif is an outer membrane protein which satisfies the following requirements: 1) to have a secretion signal which allows the fusion protein to pass through the cytoplasmic membrane; 2) to have a target signal which allows the fusion protein to anchor in the outer membrane; 3) to be expressed on the outer membrane in large quantities; and 4) to be expressed stably irrespective of protein size. Thus far, a surface-anchoring motif, which meets all of the requirements, has not yet been developed.

Meanwhile, lipoproteins have also been used as a surface-anchoring motif. Especially, lipoprotein from *E. coli* is very useful, because it translocates across

the inner membrane by the N-terminal secretion signals and directly linked to outer or inner membrane lipids via the covalent bond of its terminal L-cystein. Lpp, a major lipoprotein from *E. coli*, which is associated with the outer membrane at its N-terminus and with the cell wall peptidoglycan (PG) at its C-terminus, can be used to secrete and transport foreign proteins onto the surface of *E. coli* by fusion with the outer membrane protein A (OmpA, Francisco et al., *Proc. Natl. Acad. Sci. USA*, 1992, 489, 2713-2717). Another lipoprotein used in the surface expression of foreign protein is TraT. It was reported that TraT has been used to express peptides such as polioviral C3 epitope on the cell surface of *E. coli* (Felici et al., *J. Mol. Biol.*, 1991, 222, 301-310). Additionally, a peptidoglycan-associated lipoprotein (PAL), whose function has not been elucidated clearly yet, was used for the surface expression of a recombinant antibody (Fuchs et al., *Bio/Technology*, 1991, 9, 1369-1372). In this case, C-terminus of the PAL was associated with peptidoglycan and N-terminus of it was fused to the recombinant antibody exposed on the cell surface.

Secretory proteins, which pass through the outer membrane, may be used as the surface anchor, but these are not well developed in Gram-negative bacteria. Only a few proteins have the secretory mechanisms by aid of

the helper proteins. For instance, the lipoprotein pullulanase secreted from *Klebsiella oxytoca* is anchored on the outer membrane via a linkage between the lipid and its N-terminus, and completely secreted into a culture medium during the growth-resting phase. Kornacker et al. have been tried to express β-lactamase on the cell surface using the N-terminal fragment of pullulanase, but the expressed pullulanase-β-lactamase fusion protein was released to the cell media after short period of anchoring. When using alkaline phosphatase, a periplasmic space protein, as a target protein, the surface expression was not achieved. Functional expression of alkaline phosphatase appears to be difficult because at least 14 proteins are necessary for the secretion of this protein (Kornacker et al., *Mol. Microl.*, 1990, 4, 1101-1109).

The IgA protease derived from *Neisseria*, a pathogenic microorganism possessing an interesting secretion system, has a secretion signal at the C-terminal beta-domain and this signal guides the N-terminal protease domain onto the cell surface. This protease is secreted into the culture medium by its own catalytic hydrolysis after being anchored on the outer membrane. Using the IgA protease beta-subunit, the 12kDa form of cholera toxin B-subunit was surface

expressed (Klauser et al., *EMBO J.*, 1990, 9, 1991-1999). However, the protein folding occurred in the periplasmic space during the transport prevents the secretion of the fusion protein.

Proteins from cell surface organelles of Gram-negative bacteria, such as flagella, pili, and fimbriae, may be also available as surface anchoring motifs. For example, a flagellin, the subunit protein of flagellar filament, was used for the stable surface expression of cholera toxin B subunit and a B-type hepatitis viral peptide, and which were found to strongly react with their corresponding antibodies (Newton et al., *Science*, 1989, 244, 70-72). When using fimbrilin, a fimbria subunit protein, as a surface anchoring motif, only small size peptides were successfully expressed (Hedegaard et al., *Gene*, 1980, 85, 115-124).

Similar attempts have recently been developed in Gram-positive bacteria using surface proteins of Gram-positive and negative bacteria as surface anchoring motifs (Samuelson et al., *J. Bacterial.*, 1995, 177, 1470-1476). A malaria blood stage antigen consisting of 80 amino acid residues and a albumin-associated protein from *Streptococcus* protein G were effectively expressed on the cell surface of Gram-positive bacteria using a surface expression system containing

the *Staphylococcus aureus*-derived Protein A as a surface anchoring motif and a secretion signal from *Staphylococcus hyicus*-derived lipase.

As a result of extensive research on surface expression in Gram-positive and Gram-negative bacteria, various surface expression systems are developed and patented in the U. S. A., Europe, and Japan. In the past three years, eight patents concerning surface expression systems have been issued, among which, the case using outer membrane proteins of Gram-negative bacteria is five (WO9504069, WO9324636, WO9310214, EP603672 and US5356797), using a pilus, a cell surface organelle, is one (WO9410330), and using a cell surface lipoprotein is one (WO9504079).

The most widely used bacterial host for foreign protein production is *E. coli* because it is easy to culture and its gene structure is well known. Foreign proteins, however, are not well secreted into culture media under ordinary conditions of *E. coli*. Additionally, when foreign proteins are excessively expressed, they are accumulated as inclusion bodies within the cell. Accordingly, the purification of them requires a refolding process for solubilizing the inclusion bodies, which results in a significant reduction in yield. Further, since *E. coli* produces endotoxins harmful to the human body, the recombinant

proteins may be contaminated with the toxins when being purified.

In contrast, yeast has been studied as a host for producing useful foreign proteins by genetic engineering techniques because it can easily secrete proteins in active forms into the culture media under the control of its own intracellular secretion system, which is operated in a manner similar to that of higher eucaryotic organisms.

Since the production of interferon in 1981 (Hitzeman et al., 1981, *nature*, 293; 717-722), yeast has been extensively utilized for the production of foreign proteins. In addition, not only were recombinant proteins of yeast approved by the FDA of the U. S. A. for their safety to the human body, but also most of the regulatory mechanisms of gene expression in yeast are known (Strathern et al., *The Molecular Biology of the Yeast Saccharomyces, Metabolism and Gene Expression*, Cold Spring Harbor Laboratory, N. Y., 1982). Accordingly, yeast system provides several significant advantages for the production of foreign proteins. For example, the proteins expressed in yeast are safe to the human body, and extracellularly secreted in conformations retaining high specific activity, similar to those expressed in animal or human cells. Furthermore, the

purification processes for the proteins produced from yeast is simple compared to *E. coli*, and requires no refolding processes to obtain active forms, thus showing high production yield. Particularly, the surface expression in *S. cerevisiae* has recently been under extensive study. A few years ago, studies on the surface expression of foreign proteins in yeast were mainly focused on α-agglutinin, a typical cell wall protein as a surface anchoring motif (Schreuder et al., *Yeast*, 1993, 9, 399). In recent years, the study about surface anchoring motif has been extended to various cell wall proteins. Above all, the screening of surface anchor proteins through the conserved sequence analysis is extensively achieved, as the genome project for *S. cerevisiae* has been completed (Hamada et al., *Mol. Gen. Genet.*, 1998, 258, 53). Using such surface proteins as surface anchoring motifs, various enzymes, including α-galactosidase, glucoamylase, lipase, and cutinase have been stably expressed on the cell surface of *S. cerevisiae*. In addition, expression of various enzymes on cell surface could develop many useful industrial biocatalysts (Murai et al., *Appl. Microbiol. Biotechnol.*, 1999, 51, 65). A surface expression system using α-agglutinin as an anchoring motif has been developed and commercialized by Invitrogen Corporation. Furthermore, yeast, which is a

eucaryotic microorganism usually harmless to the human body, is highly useful as a host for producing proteins for use in food or medical materials. For instance, B-type hepatitis viral antigen (HbsAg) was expressed on the yeast cell surface with the aim of developing live vaccines (Schreuder et al., *Vaccine*, 1996, 14, 383).

As mentioned above, active research has been directed to cell wall proteins throughout the world, but limited to *S. cerevisiae*. At present, researches on the cell wall proteins of other yeasts, such as *Candida albicans*, are in the initial stage. Therefore, there remains an urgent need for studies on surface proteins and surface expression thereof in industrially useful yeasts. Because the mediating proteins that have been studied thus far make use of glycosylphosphatidylinositol-anchor for surface anchoring, the proteins of interest must be linked to the anchor proteins at their carboxyl termini. However, some of the proteins may not exhibit their full activity under such a condition, and this is recognized as a drawback in yeast surface expression system.

SUMMARY OF THE INVENTION

Leading to the present invention, the intensive and thorough research on extracellular transport of exogenous proteins, conducted by the present inventors, resulted in the finding that surface proteins derived from *Hansenula polymorpha*, an industrially useful methanol-assimilating yeast, are highly effective in construction of surface expression systems and development of biocatalyst application systems. The surface expression system established in the present invention can stably express proteins of interest onto the cell surface, thus finding numerous applications in various fields, including immobilization of biocatalysts and large-scale production of proteins, such as enzymes, antigens, antibodies, etc.

Therefore, it is an object of the present invention to resolve the foregoing or other problems encountered in prior arts and to provide systems for the exportation of exogenous polypeptides to discrete regions of a host cell in which they are expressed, and uses thereof.

It is another object of the present invention to provide a novel surface anchor protein and its gene, derived from *Hansenula polymorpha*.

It is a further object of the present invention to provide a surface expression system using all or part of the surface proteins as mediators for surface

expression of exogenous polypeptides or proteins.

It is still a further object of the present invention to provide an expression mediator protein, isolated form *Hansenula polymorpha*, which can be fused to the amino termini of proteins to be expressed on the cell surface, and a gene encoding the mediator protein.

It is still another object of the present invention to provide a surface expression system in which all or part of the expression mediator protein which can be fused to the amino termini of proteins to be expressed, is employed.

It is still another object of the present invention to provide a surface expression system in which all or part of the known surface protein derived from *Saccharomyces diastaticus* and *Hansenula polymorpha*, is employed.

In accordance with an aspect of the present invention, there are provided the isolated DNA sequences, containing the base sequence *HpSED1* represented by the SEQ. ID. NO: 1, the base sequence *HpRIR2* represented by the SEQ. ID. NO: 4, the base sequence *HpGAS1* represented by the SEQ. ID. NO: 5, the base sequence *HpTIP1* represented by the SEQ. ID. NO: 6, and the base sequence *HpCWP1* represented by the SEQ. ID. NO: 7, all encoding novel cell wall proteins of

Hansenula polymorpha, and their DNA homologues.

In accordance with another aspect of the present invention, there are provided novel *E. coli* strains harboring recombinant vector containing a 4 kb EcoRI fragment of the base sequence *HpSED1*, a 5.5 kb SalI fragment of the base sequence *HpPIR2*, a 3 kb PstI fragment of the base sequence *HpGAS1*, a 3.5 kb XbaI fragment of the base sequence *HpTIP1*, and a 6 kb SalI fragment of the base sequence *HpCWP1*.

In accordance with a further aspect of the present invention, there are provided surface expression systems in which base sequences of *HpSED1*, *HpPIR2*, *HpGAS1*, *HpTIP1*, *HpCWP1*, *STA1* from *Saccharomyces diastaticus*, *HpWSC1* from *Hansenula polymorpha* and *WSC1 from Saccharomyces cerevisiae* or their partial fragments are used to express mediator proteins which locate exogenous proteins onto the surface of a cell. Herein, useful in the present invention is a eukaryotic cell selected from the group consisting of yeast *spp.*, including *Candida spp.*, *Debaryomyces spp.*, *Hansenula spp.*, *Kluyveromyces spp.*, *Pichia spp.*, *Schizosaccharomyces spp.*, *Yarrowia spp.*, *Saccharomyces spp.*; and *mold spp.* including *Aspergillus spp.*, *Penicillium spp.*, and *Rhizopus spp.*

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a photograph showing Southern blot analysis results of DNA fragments obtained by treating the genome of *Hansenula polymorpha* with BamHI (lane 3), EcoRI (lane 4), PstI (lane 5), and XbaI (lane 6), along with a EcoRI fragment of the *Saccharomyces cerevisiae* genome (lane 1) and a marker (lane 2) after the DNA fragments are hybridized with a *SED1* gene as a probe.

Fig. 2a is a photograph showing Southern blot analysis results of DNA fragments obtained by treating the genome of *Hansenula polymorpha* with ClaI (lane 1), EcoRI (lane 2), HindIII (lane 3), SalI (lane 4) and XhoI (lane 6), along with a marker (lane 6) and an EcoRI fragment of the *Saccharomyces cerevisiae* (lane 7) after the DNA fragments are hybridized with a *CWP2* gene as a probe.

Fig. 2b is a photograph showing Southern blot analysis results of DNA fragments obtained by treating the genome of *Hansenula polymorpha* with BamHI (lane 1),

ClaI (lane 2), EcoRI (lane 3), HindIII (lane 4), PstI (lane 5), XbaI (lane 6), and XhoI (lane 7), along with a marker (lane 8) and an EcoRI fragment of *Saccharomyces cerevisiae* genome after the DNA fragments are hybridized with a *TIR1* gene as a probe.

Fig. 3 is a photograph showing Southern blot analysis results of DNA fragments obtained by treating the genome of *Hansenula polymorpha* with ClaI (lane 1), HindIII (lane 2), SalI (lane 3), XhoI (lane 4), BamHI (lane 7), EcoRI (lane 8), PstI (lane 9), and XbaI (lane 10), along with a marker (lane 5 and 6), after the DNA fragments are hybridized with a GAS1 gene as a probe.

Fig. 4 is a photograph showing Southern blot analysis results of DNA fragments obtained by treating the genome of *Hansenula polymorpha* with BamH1 (lane 1), EcoRI (lane 2), ClaI (lane 3), PstI (lane 4), XbaI (lane 5), XhoI (lane 6), EcoRI (lane 9), XbaI (lane 10), XhoI (lane 11), along with a marker (lanes 7 and 12) and an EcoRI fragment of the *Saccharomyces cerevisiae* genome (lane 8), after the DNA fragments are hybridized with a TIP1 gene as a probe.

Fig. 5 is a photograph showing Southern blot analysis results of DNA fragments obtained by treating the genome of *Hansenula polymorpha* with ClaI (lane 1), HindIII (lane 2), PstI (lane 3), SalI (lane 4), and

XhoI (lane 5), after the DNA fragments are hybridized with a CWP1 gene as a probe.

Fig. 6 is a schematic diagram showing a surface expression vector for expressing CMCase on the cell surface of *Hansenula polymorpha*, which contains a GAPDH promoter (pGAPDH), a killer toxin signal sequence (KTsig) and a CMCase gene with a termination codon (Term).

Fig. 7 is a histogram showing the CMCase activity detected on the cell surface of *Hansenula polymorpha* according to surface expression mediators.

Fig. 8 shows graphs in which the CMCase activity detected on cell surfaces of *Hansenula polymorpha* strains, which have surface expression mediators is plotted with respect to culture time period.

Fig. 9 is a schematic diagram showing a surface expression vector for expressing the protein HpPir2p on the cell surface of *Hansenula polymorpha*, which contains a GAPDH promoter (pGAPDH), a killer toxin signal sequence (KTsig) and a CMCase gene with a termination codon (Term), and a full length of a PIR2 gene (Pir-N) fused to the amino terminus of the killer toxin signal sequence (KTsig) or a partial fragment of a PIR2 gene (Pir-C) fused to the carboxy terminus of the CMCase gene.

Fig. 10a is a schematic diagram showing domains

of the protein Wsc1p and their cellular location sites.

Fig. 10b is a schematic diagram showing surface expression vectors for expressing exogenous proteins on the cell surface of *Hansenula polymorpha*, in which several truncated *HpWSC1* gene fragments, devoid of either the cystein motif or the transmembrane domain, and of both of them, and an intact *HpWSC1* gene encoding the full length of HpWsc1p, are inserted.

Fig. 11 is a photograph showing the glucose oxidase activity measured according to the plate activity assay method from *Hansenula polymorpha* strains which use expression mediators (Tip40, GasF, CwpF and Wsc-ct) and no expression mediators (GOD*).

Fig. 12 shows FACS analysis results of *Hansenula polymorpha* strains (white) on which glucose oxidase is expressed by employing surface expression mediators such as CwpF (panel A). Tip40 (panel B), GasF (panel C), and Wsc-ct (panel D), along with a wild type (gray).

Fig. 13 is a schematic diagram showing surface expression systems for expressing exogenous proteins on the cell surface of *Saccharomyces cerevisiae* by use of the gene *STA1*, which are constructed with vectors comprising the signal sequence only (STASS), both of the signal sequence and the octapeptide sequence (STASS-8), all of the signal sequence, the octapeptide

sequence and the threonine/serine-rich domain (STASS-8-TS), both of the signal sequence and the threonine/serine-rich domain (STASS-TS), both of the signal sequence and alpha-agglutinin (STASS-CMC-Agalc), and all of the signal sequence, the octapeptide, and alpha-agglutinin (STASS-8-CMC-Agalc), along with a CMCase gene as a reporter gene.

Fig. 14 is a photograph showing the CMCase activity measured according to the plate activity assay method from *Saccharomyces cerevisiae* strains which harbor the signal sequence only (SS), both of the signal sequence and the octapeptide sequence (SS-8), all of the signal sequence, the octapeptide sequence and the threonine/serine-rich domain (SS-8-TS), and both of the signal sequence and the threonine/serine-rich domain (SS-TS).

Fig. 15 shows FACS analysis results of *Saccharomyces cerevisiae* strains on which CMCase is expressed by employing surface expression vectors (gray), along with a wild type (white).

DETAILED DESCRIPTION OF THE INVENTION

Established in the present invention are systems for the export of exogenous polypeptides to discrete regions of a yeast cell in which it is expressed,

which can be used for regenerable whole-cell biocatalyst.

To circumvent the problems associated with *Saccharomyces cerevisiae*, which has been used for the industrial production of recombinant proteins, *Hansenula polymorpha*, a methylotrophic yeast like *Pichia pastoris*, is studied for the expression of recombinant proteins. Because the cell wall structure of *Hansenula polymorpha* is not yet known, genes, which encode proteins mediating the exportation of exogenous proteins to the cell surface, are screened and cloned in the present invention. In addition, the cloned genes are fused to genes coding for proteins of interest to construct novel surface expression systems capable of expressing exogenous proteins on yeast cell surfaces.

Therefore, the present invention pertains to genes encoding cell wall proteins of *Hansenula polymorpha*.

Cell wall proteins of *Hansenula polymorpha* were identified to be very similar in characteristics to those of *Saccharomyces cerevisiae*. Genes coding for Cwp1p (GenBank D37975, van der Vaart et al., J. Bacteriol., 1995, 177, 3104), Cwp2p (GenBank Z28096, Van der Vaart et al., *J. Bacteriol.*, 1995, 177, 3104), Sed1p (GenBank X66838, Seidel, J. and W. Tanner, *Yeast*,

1997, 13, 3104), Tir1p (GenBank X12775), and Tip1p (GenBank M71216, Van der Vaart et al., *J. Bacteriol.*, 1995, 177, 3104), all known as cell wall proteins, and Gas1p (GenBank X53424, Benghezal et al., *J. Cell Biol.*, 1995, 130, 1333) known as a cytoplasmic membrane protein, were used to search for corresponding ones from the genome of *Hansenula polymorpha*.

(1) Isolation of *HpSED1* Gene of *Hansenula polymorpha*

Encoded by the gene *SED1*, 1,017 bp long, Sed1p is a protein which consists of 338 amino acid residues and a predominant protein on the cell wall of *Saccharomyces cerevisiae* in the stationary phase.

Using a *SED1* gene segment of *S. cerevisiae* as a probe, a 4 kb EcoRI fragment was obtained from the genome of *Hansenula polymorpha* (see Fig. 1) and inserted into the vector. The *SED1* homologous gene fragment of *Hansenula polymorpha* named as *HpSED1*, was identified to have the base sequence represented by the SEQ. ID. NO: 1, which codes for an open reading frame consisting of 131 amino acid residues represented by the SEQ. ID. NO: 11. This *HpSED1* gene fragment of *Hansenula polymorpha* was much shorter than the *SED1* gene of *S. cerevisiae*. However, the HpSed1p encoded by the *HpSED1* gene of *Hansenula polymorpha* has

the same amino acid repeating sequences, shows a similar threonine/serine-rich structure, and shares homology of 58.4% in amino acids sequence with *S. cerevisiae*. In addition, the HpSed1p of *Hansenula polymorpha* was seemed to have a signal sequence consisting of 17 amino acids, and a putative GPI-anchor signal at its carboxyl terminus. A novel *E. coli* transformant harboring a recombinant vector containing the 4 kb EcoRI fragment of the *HpSED1* gene was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0825BP on July 11, 2000.

(2) Isolation of *HpPIR2* Gene of *Hansenula polymorpha*

Encoded by the *TIR1* gene, 765 bp long, Tir1p is a protein found at the cell well of *S. cerevisiae*, consisting of 254 amino acids. The protein shows hyper N-glycosylation and is rich in serine/alanine and well expressed under the anaerobic condition.

Using a *TIR1* gene fragment of *S. cerevisiae* as a probe, a 5 kb ClaI fragment was obtained from the genome of *Hansenula polymorpha* (see Fig. 2b) and inserted into a vector. DNA sequence analysis revealed that the ORF in the ClaI fragment shares homology of

51.2% with the *PIR2* gene of *S. cerevisiae* (GenBank D13741, Toh-e et al., *Yeast*, 1993, 9, 481).

Cwp2p, consisting of 92 amino acids, is known as a most effective surface anchor except α-agglutinin, among the proteins of *S. cerevisiae* (van der Vaart et al., *Appl. Environ. Microbiol.*, 1997, 63, 615). In the present invention, a 5.5 kb SalI fragment obtained from the genome of *Hansenula polymorpha* using a *S. cerevisiae CWP2* gene as a probe (see Fig. 2a), was identified as the same fragment with the ClaI fragment cloned by *TIR1* by DNA sequencing. Both proteins, Cwp2p and Tir1p, were reported to contain the repeating amino acid sequences represented by the SEQ. ID. NO: 2, called PIR1/2/3 repeat which made strong homology with Pir2p protein.

The Pir2p of *S. cerevisiae* coded by *PIR2* is known to be released from the cell wall by alkali treatment, usually have a Kex2 cleavage site, also this protein is anchored on the cell wall in a form different from that of the GPI (glycosylphosphatidylinositol)-anchor protein and is not released by glucanase treatment (Toh-e et al., *Yeast*, 1993, 9, 481). The N-terminal amino acids analysis of major cell wall protein released by alkali treatment from *Hansenula polymorpha* cell wall, showed the same amino acids sequence with the HpPir2p polypeptide following the kex2p cleavage

site, represented by the SEQ. ID. NO: 3. Thus, the *HpPIR2* gene obtained from *Hansenula polymorpha* was expected to be a gene coding for the HpPir2p, which located in the cell wall. The HpPir2p of *Hansenula polymorpha* is encoded by a 1,014 bp gene represented by the SEQ. ID. NO: 4, consisting of 337 amino acids with *PIR1/2/3* repeat. This protein possessed a secretion signal sequence consisting of 18 amino acids and a Kex2p cleavage site at amino acid residue 68. A novel *E. coli* transformant harboring a recombinant vector containing the 5.5 kb SalI fragment of the *HpPIR2* gene of *Hansenula polymorpha* was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0827BP on July 11, 2000.

(3) Isolation of *HpGAS1* gene of *Hansenula polymorpha*

Encoded by the *GAS1* gene, 1,680 bp long, Gas1p consists of 559 amino acids and is linked to the cell membrane of *Saccharomyces cerevisiae*. However, its amino terminal domain is exposed to the exterior, penetrating the cell wall.

In the present invention, using a *GAS1* gene fragment of *S. cerevisiae* as a probe, a 3 kb PstI

fragment was obtained from the genome of *Hansenula polymorpha* (see Fig. 3) and inserted into a vector. DNA sequence analysis revealed an ORF shares 70.7% homology with the Gas1p of *S. cerevisiae* and this was named as HpGas1p. HpGas1p of *Hansenula polymorpha* consists of 537 amino acids represented by the SEQ. ID. NO: 14, which is encoded by the gene represented by the SEQ. ID. NO: 5, 1,614 bp long. The Gas1p of *Hansenula polymorpha* also possesses a secretion signal sequence consisting of 18 amino acids, but GPI-anchor signal was different with that of *S. cerevisiae*. A novel *E. coli* transformant harboring a recombinant vector containing 3 kb PstI fragment of the *HpGAS1* gene of *Hansenula polymorpha* was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0828BP on July 11, 2000.

(4) Isolation of *HpTIP1* Gene of *Hansenula polymorpha*

Encoded by the 633 bp, *TIP1* gene of *Saccharomyces cerevisiae*, Tip1p is a GPI-anchor protein consisting of 210 amino acids with high homology with Tir1p and the expression of Tip1p is induced by cold shock.

In the present invention, using a *TIP1* gene fragment of *S. cerevisiae* as a probe, a 3.5 kb XbaI

fragment was obtained from the genome of *Hansenula polymorpha* (see Fig. 4) and inserted into a vector. DNA sequence analysis revealed an ORF consisting with 852 bp sequence represented by the SEQ. ID. NO: 6, coding for 283 amino acids represented by the SEQ. ID. NO: 13. A novel *E. coli* transformant harboring a recombinant vector containing the 3.5 kb XbaI fragment of the *HpTIP1* gene of *Hansenula polymorpha* was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0824BP on July 11, 2000.

(5) Isolation of *HpCWP1* Gene of *Hansenula polymorpha*

Cwp1p is a typical GPI-anchor protein, which is encoded by the 720 bp, *CWP1* gene of *Saccharomyces cerevisiae*, consisting of 239 amino acids.

In the present invention, using the full *CWP1* gene of *S. cerevisiae*, a 6 kb SalI fragment was obtained from the genome of *Hansenula polymorpha* (see Fig. 5) and cloned into a vector. DNA sequence analysis revealed a putative ORF with 246 bp sequence represented by the SEQ. ID. NO: 7, which was named as *HpCWP1*, even though this protein showed low homology with the *CWP1* of *S. cerevisiae*. Additionally, the

HpCwp1p represented by the SEQ. ID. NO: 15, was deduced to have a secretion signal consisting of 15 amino acids, also the putative GPI-anchor signal. A novel *E. coli* transformant harboring a recombinant vector containing 6 kb SalI gene fragment coding for the HpCwp1p of *Hansenula polymorpha* was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0826BP on July 11, 2000.

(6) Isolation of *HpWSC1* Gene of *Hansenula polymorpha*

Wsc1p is a protein responsible for stress response, anchored in the cell membrane of *Saccharomyces cerevisiae*. This stress response protein was reported to have a carboxy-terminal transmembrane domain, a serine/threonine-rich domain traversing the cell wall, and an amino-terminal cystein motif which is exposed to the exterior, functioning to detect external signals (Verna et al., *Proc. Natl. Acad. Sci. USA*, 1997, 94, 13804).

Based on the fact that fluorescence was observed on the cell surface when a green fluorescence protein (GFP) was expressed as a fusion partner with Wsc1p, it was deduced that the amino terminal domain of Wsc1p

could be used as a surface-expression mediator. In the process of isolating the *LEU2* gene of *Hansenula polymorpha*, the *HpWSC1* gene was obtained by Agaphonov et al. (Agaphonov et al., *Yeast*, 1994, 10, 509, GenBank U00889). DNA sequencing analysis disclosed that the *HpWSC1* gene of *Hansenula polymorpha* consists of the 1,110 bp sequence represented by the SEQ. ID. NO: 8 encoding the 373 amino acid sequence. The protein encoded by the *HpWSC1* gene isolated from *Hansenula polymorpha* was assumed as a cell surface protein different in anchoring mechanism from GPI-anchor proteins and could be used to develop surface expression systems by fusion to the amino terminal domain of foreign proteins.

In accordance with the embodiment of the present invention, there are provided *Hansenula polymorpha*-derived cell wall protein genes, *HpSED1*, *HpPIR2*, *HpGAS1*, *HpTIP1*, and Hp*CWP1*, and a membrane protein gene *HpWSC1* gene, vectors containing them, and cells transformed with the vectors.

Also, the present invention pertains to surface expression systems using novel cell wall proteins isolated from *Hansenula polymorpha* as mediators for surface expression of foreign proteins.

In accordance with another embodiment of the present invention, there are provided surface

expression systems using GPI-anchor proteins isolated from *Hansenula polymorpha*, as mediators for surface expression of foreign proteins.

Expected to be useful as mediator genes for surface expression systems, four putative GPI-anchor protein genes are cloned from *Hansenula polymorpha*. Along with each of the mediators, carboxyl methyl cellulase (hereinafter referred to as "CMCase") derived from *Bacillus subtilis* (Park et al., *Agric. Biol. Chem.*, 1991, 55, 441) is used in the surface expression systems as a reporter protein, in accordance with the present invention.

In this regard, a GAPDH promoter (Sohn et al., *Appl. Microbiol. Biotechnol.*, 1999, 51, 800) and a killer toxin signal sequence (Sor, F. and Fukuhara, *Curr. Genet.*, 1985, 9, 147) were inserted into an expression vector. Then, the CMCase gene was fused in frame to construct a CMCase surface expression vector, which can express CMCase under the control of the GAPDH promoter. Four putative GPI-anchor protein genes, *HpCWP1*, *HpGAS1*, *HpTIP1* and *HpSED1* gene fragments, are individually inserted to the expression vector at a carboxy terminal region of the CMCase to construct a CMCase surface expression vector. Based on the fact that a GPI-anchor motif located within the 40 amino acids of carboxy terminus (van der Vaart et al., *Appl.*

Environ. Microbiol., 1997, 63, 615), CMCase surface expression vectors were also constructed with a nucleic acid segment encoding the carboxy-terminal 40 amino acids of each mediators. After all of the surface expression vectors were introduced to *Hansenula polymorpha* (Hill et al., *Nucl. Acids Res.*, 1991, 19, 5791), the transformants thus obtained were cultured and the CMCase activity of them was measured in the supernatant and the whole cell fraction.

In whole cell fractions, the expression vectors in which each of the mediator genes was inserted exhibit significantly improved CMCase activity compared to control vectors in which the signal sequence is absent or no mediator genes are fused, demonstrating that CMCase is exported to the cell surface by each mediator (see Table 1). Especially, HpTip1p, HpGas1p and HpCwp1p were found to translocate CMCase to the cell surface at higher efficiency than other mediators (see Fig. 7).

While the CMCase activity was decreased in most of the cells when they reached the stationary phase, the CMCase activity of the cells using HpCwp1p as a surface anchor were found to remain high throughout the culturing period, although being lowered in the stationary phase. Therefore, HpCwp1p was inferred to be the most stable mediator among the proteins tested

(see Fig. 8).

In accordance with a further embodiment of the present invention, there are provided surface expression systems using non GPI-anchor proteins isolated from *Hansenula polymorpha* as mediators for surface expression of foreign proteins.

In one version of this embodiment, an *HpPIR2* gene, which encodes a cell wall protein different from GPI-anchor proteins were used for constructing a surface expression system.

In contrast to GPI-anchor proteins, the protein Pir2p is anchored in the cell wall through the hyper O-glycosylation of its serine/threonine-rich domain with the glycans present in the cell wall. Thus, the protein Pir2p is not released from the cell wall by the enzymes, which hydrolyze glycans only, but released from the cell wall by alkaline treatment, which cleaves all glycosylation. In the present invention, surface expression systems utilizing carboxy- and amino-terminal regions of the protein HpPir2p were constructed. To evaluate the anchoring ability of the mediators derived from HpPir2p, CMCase, the reporter, activity was measured (see Fig. 9).

Almost the same CMCase activity was obtained when the carboxy-terminal region of the protein HpPir2p was expressed as a mediator as when CMCase gene without

mediator was expressed. By contrast, where the amino-terminal region of the protein HpPir2p was used as an anchoring mediator, CMCase activity was detected not only in the supernatant, but also in the whole cell fraction (see Table 2). It was reported that the protein Pir2p was secreted to the culture medium by the overexpression thereof in vivo (Russo et al., *Proc. Natl. Acad. Sci. USA*, 1992, 89, 3671). Therefore, the CMCase activity detected in the whole cell fraction was attributed by the enzyme anchored in the cell wall.

Therefore, the protein HpPir2p can be anchored in the cell wall and be used as a surface expression mediator. Over conventional GPI-anchor proteins, the protein Pir2p has the advantage of linking to an amino terminal of target protein to its carboxy terminus.

In another version of the embodiment, the *WSC1* gene, which encodes a transmembrane protein different from GPI-anchor proteins, was used to construct a surface expression system.

The protein Wsc1p is composed of a transmembrane domain by which the protein is anchored in the cell membrane, a serine/threonine-rich domain that traverses the cell wall, and an amino-terminal cystein motif which exists in the extracellular space (Verna et al., *Proc. Natl. Sci. USA*, 1997, 13804), as illustrated in Fig. 10a.

In order to establish a surface expression system using the HpWsc1p, several truncated *HpWSC1* gene fragments, devoid of either the cystein motif or the transmembrane domain and of both of them were inserted in the CMCase expression vector (see Fig. 10b) and the CMCase activity was analyzed.

Only where the transmembrane domain was presented in the fusion protein, the CMCase was not secreted, but rather attached to the cell. However, although the transmembrane domain was present, CMCase activity was not detected in the whole cell fraction unless the cystein motif is present. This result might come from the fact that the CMCase was not sufficiently exposed on the cell surface. In contrast, where the transmembrane domain was absent, the CMCase activity was mostly detected in the supernatant. In addition, the cystein motif made an additional contribution to the secretion of the CMCase (see Table 3).

Therefore, the mediator derived from the HpWsc1p must have the cystein motif and the transmembrane domain, both, for anchoring efficiency. In the present invention, the HpWsc1p was suggested as a novel mediator for surface expression of proteins.

In accordance with still a further embodiment of the present invention, there are provided surface expression systems for exporting glucose oxidase to

the cell surface by use of the HpTip1p, HpGas1p and HpCwp1p, and the HpWsc1p.

Glucose oxidase, a flavoenzyme derived from *Aspergillus niger*, consists of two identical polypeptide chain subunits. Upon being expressed in *Hansenula polymorpha*, this enzyme is known to undergo hyper glycosylation.

When being expressed in the presence of the anchoring mediators such as HpTip1p, HpGas1p, HpCwp1p and HpWsc1p, glucose oxidase was found to form smaller activity circles on plates (Hodgkins et al., *Yeast*, 1993, 9, 625) than the secreted ones (see Fig. 11).

In all cases of using the anchoring mediators, fluorescent cells were observed by FACS (fluorescence activated cell sorter) analysis, indicating that the glucose oxidase was expressed and anchored onto the cell surface. Additionally, higher glucose oxidase activity was detected in the whole cell fraction than in the supernatant throughout all of strains. When HpCwp1p was employed, fluorescence was detected from a large number of cells, demonstrating that HpCwp1p had an excellent cell wall anchoring activity. It was also found that the 40 amino acids fragment of HpTip1p was efficient in expression of proteins onto the cell surface (see Fig. 12). Under the mediation of HpGas1p and HpWsc1p, fluorescence, which proves the cell wall

anchoring activity, was detected, but in a low level. However, even in this case, there seemed to be no problems in detecting the activity of the enzyme if the substrate penetrated the cell wall.

Accordingly, not only is HpCwp1p found to be the most effective surface expression mediator among the GPI-anchor proteins, but also the proteins HpPir2p and HpWsc1p can be suggested as novel expression mediators in accordance with the present invention.

Herein, useful in the present invention is a eukaryotic cell selected from the group consisting of yeast spp., including *Candida* spp., *Debaryomyces* spp., *Hansenula* spp., *Kluyveromyces* spp., *Pichia* spp., *Schizosaccharomyces* spp., *Yarrowia* spp., *Saccharomyces* spp.; and *mold* spp. including *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp.

In accordance with still another embodiment of the present invention, there are provided surface expression systems using the *Saccharomyces diastaticus*-derived glucoamylase gene *STA1*.

With the 2,337 bp sequence represented by the SEQ. ID. No: 9, the glucoamylase derived from *Saccharomyces diastaticus* is secreted to extracellular spaces to hydrolyze extracellular starch. The amino acid sequence analysis of this enzyme showed a signal

sequence (SS) in the amino-terminal region and threonine/serine-rich (TS-rich) domain which containing the threonine and serine residues in an amount of 55% of the total amino acid residues. The signal sequence and the threonine/serine-rich domain are linked to each other via the octapeptide represented by the SEQ. ID. NO: 10. While this octapeptide plays a role in directing the protein to the exterior, the threonine/serine-rich domain is responsible for the support of the proteins upon its penetration through the cell wall, thus acting like an anchoring mediator (Venturini et al., *Mol. Microbiol.*, 1997, 23, 997; Yamashita, I., *Agric. Biol. Chem.*, 1989, 53, 483; Yamashita et al., *Agric. Biol. Chem.*, 1984, 48, 1611).

With reference to Fig. 13, there are shown vector diagrams for the surface expression systems using the *STA1* gene. As depicted, the surface expression systems are constructed with vectors containing the signal sequence only, both of the signal sequence and the octapeptide sequence, all of the signal sequence, the octapeptide sequence and the threonine/serine-rich domain, both of the signal sequence and alpha-agglutinin, and all of the signal sequence, the octapeptide, and alpha-agglutinin, along with a CMCase gene as a reporter gene.

When employing the signal sequence only, most of the CMCase was secreted extracellularly, not anchored in the cell wall, as measured by the pNPC (p-nitrophenyl β-D-cellobiocide) method (Deshpande et al., *Anal. Biochem.*, 1984, 138, 481). In contrast, when employing the threonine/serine-rich domain linked to the signal sequence via the octapeptide sequence, the CMCase activity was low in the supernatant, but high in the whole cell fraction. The same results were confirmed by plate activity assay. Large circles were formed around the colonies, which harbor the signal sequence only, indicating that the CMCase was mostly secreted outside the cells. The presence of the threonine/serine-rich domain hindered the enzyme secretion, as proven by the small circles formed around the colonies (see Table 4 and Fig. 14).

In addition, fluorescence was detected in the cells harboring the threonine/serine-rich domain by FACS analysis, suggesting the presence of CMCase on the cell surface (see Fig. 15).

When glucoamylase gene was used for the construction of surface expression systems, glucoamylase or its domains could be fused to the amino terminus of target protein. Thus, these systems could circumvent the problems arising when a carboxy-terminal active domain of the target protein was

interrupted by the mediator fused to the carboxy terminus.

Therefore, the mediators described herein allow the development of surface expression systems capable of expressing various types of target proteins, according to the characteristics of the target proteins.

EXAMPLES

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

EXAMPLE 1: Isolation of Genes Encoding Cell Wall Expression Proteins from *Hansenula polymorpha*

For use in the cloning, the strain DL1-L (Δ *leu2*), derived from *Hansenula polymorpha* DL1 (ATCC 26012) was obtained from NPO Biotechnologia (Moscow, Russia). Cell wall protein profile analysis by biotin labeling, which cannot penetrate into the cell wall, demonstrated that this strain had very similar profile of surface proteins with *Saccharomyces cerevisiae*. For

the cloning of cell wall protein genes from *Hansenula polymorpha*, the genes encoding the glucanase extractable cell wall proteins of *S. cerevisiae* were utilized.

In order to isolate the cell wall protein gene from *Hansenula polymorpha*, genes coding for Cwp1p (GenBank D37975, van der Vaart et al., *J. Bacteriol.*, 1995, 177, 3104), Cwp2p (GenBank Z28096, van der Vaart et al., *J. Bacteriol.*, 1995, 177, 3104), Sed1p (GenBank X66838, Seidel, J. and W. Tanner, *Yeast*, 1997, 13, 3104), Tir1p (GenBank X12775), and Tip1p (GenBank M71216, van der Vaart et al., *J. Bacteriol.*, 1995, 177, 3104), all known as cell wall expression proteins, and Gas1p (GenBank X53424, Benghezal et al., *J. Cell Biol.*, 1995, 130, 1333) known as a cytoplasmic membrane protein, were obtained from *S. cerevisiae*. Reportedly, these proteins can be separated from the cell wall by glucanase treatment because their carboxy termini are linked to β-1,6-glucan of the cell wall via GPI-anchor (Kapteyn et al., *Biochim. Biophys. Act.*, 1999, 1426, 373; Kolla et al., J. Biol. Chem., 1997, 272, 17762). Using these genes as probes, the genome of *Hansenula polymorpha* was subjected to southern blot analysis. No clear signals were obtained from the genomic DNA of *Hansenula polymorpha* when the Cwp1p gene of *S. cerevisiae* was used as a probe. This was inferred to

result from low homology of the gene between the two strains. In the cases of the other four genes, clear signals were observed. From the genomic library, corresponding DNA fragments were cloned.

<1-1> Isolation of *HpSED1* gene of *Hansenula polymorpha*

In order to isolate the *SED1* homologue from *Hansenula polymorpha*, the open reading frame of the *Saccharomyces cerevisiae SED1* gene was labeled according to the Klenow fragment method by a DIG labeling system (Boehringer Mannheim) and this was used as a probe for Southern blot analysis. Following hybridization at 42°C in a 30% formamide solution, signal was detected.

The southern blot analysis results are given in Fig. 1. From the EcoRI fragment, a signal was detected at a size of about 4 kb. After the elution of DNA around 4 kb from the gel, the DNA fragment was inserted to pBluescript II SK(+) at a EcoRI site to construct a library. Under the same condition, southern blot analysis was conducted with the library to obtain a recombinant vector in which a DNA fragment hybridized with the probe was inserted. The DNA sequence analysis of the recombinant vector, named *HpSED1*, revealed that the 4 kb DNA fragment has a base sequence represented by the SEQ. ID. NO: 1 with an

open reading frame consisting of 131 amino acids represented by the SEQ. ID. NO: 11. The *HpSED1* gene of *Hansenula polymorpha* was found to be far shorter than, but have the same amino acid repeating sequences as the corresponding gene of *S. cerevisiae* and show a similar threonine/serine-rich structure, sharing homology of 58.4% with Sed1p of *S. cerevisiae*. In addition, the HpSed1p of *Hansenula polymorpha* was expected to have a signal sequence consisting of 17 amino acids, and a GPI-anchor signal at its carboxyl terminus, as analyzed by the PSORT II prediction program. A novel *E. coli* transformant harboring a recombinant vector in which the 4 kb EcoR1 fragment containing the *HpSED1* gene of *Hansenula polymorpha* is inserted, was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0825BP on July 11, 2000.

<1-2> Isolation of *HpPIR2* gene of *Hansenula polymorpha*

In order to isolate the *TIR1* homologue from *Hansenula polymorpha*, the open reading frame of the *Saccharomyces cerevisiae TIR1* gene was labeled according to the Klenow fragment method by a DIG labeling system (Boehringer Mannheim) and this fragment was used as a probe for southern blot

analysis. Following hybridization at 42°C in a 30% formamide solution, signal was detected.

The southern blot analysis results are given in Fig. 2b. From the ClaI digested fragment, a signal was detected at about 5kb (lane 9). After the elution of this part from the gel, the DNA fragment was inserted to pBluescript II SK(+) at a ClaI site to construct a recombinant vector library. Using the same method as <1-1>, a recombinant vector containing a 5 kb ClaI DNA fragment hybridized with the probe was obtained. The DNA sequence analysis of this 5 kb ClaI DNA fragment, revealed an ORF that the shares homology of 51.2% with the Pir2p of *S. cerevisiae*.

To clone the *CWP2* homologue from *H. polymorpha*, the open reading frame of the *S. cerevisiae CWP2* gene was labeled according to the Klenow fragment method by a DIG labeling system and used as a probe. Southern hybridization was conducted at 42°C in a 30% formamide solution to detect signals. The southern blot analysis results are shown in Fig. 2a. From a 5.5 kb SalI fragment, an apparent signal was detected. This 5.5 kb SalI fragment was identified to have the same DNA sequence with the ClaI fragment, as analyzed by DNA sequencing. Both of the proteins, Cwp2p and Tir1p, were found to contain the repeating amino acid sequence represented by the SEQ. ID. NO: 2, called

PIR1/2/3 repeat. It was inferred that these repeating sequences might share high homology with the *HpPIR2* gene of *Hansenula polymorpha*, thus resulting in the hybridization of the *HpPIR2* gene with the probe. To exclude this possibility, each gene deprived of the repeating sequences was used as a probe for southern blot analysis, but no clear signals were observed.

Encoded by the *PIR2* gene isolated from *S. cerevisiae*, the Pir2p protein was reported to be released from the cell wall by alkali treatment and not released by glucanase treatment, which meant that this protein anchored onto the cell wall in a different form from that of the GPI (glycosyl-phosphatidylinositol)-anchor protein, also it was known that this protein had a Kex2 cleavage site (Toh-e et al., Yeast, 1993, 9, 481). The N-terminal amino acids analysis of major cell wall protein released by by 30 mM sodium hydroxide (NaOH) from *Hansenula polymorpha* cell wall (Mrsa et al., Yeast, 13, 1145-1154, 1997), showed the same amino acids sequence with the HpPir2p polypeptide following the kex2 cleavage site, represented by the SEQ. ID. NO: 3. Thus, the gene *HpPIR2* obtained from *Hansenula polymorpha* was inferred to code for the protein HpPir2p, which translocates to the cell wall.

The Pir2p protein of *Hansenula polymorpha* was

encoded by a 1,014 bp gene, represented by the SEQ. ID. NO: 4, consisting of 337 amino acids with PIR1/2/3 repeats, represented by the SEQ. ID. NO:12. This protein possesses a secretion signal sequence consisting of 18 amino acids and a Kex2 cleavage site at amino acid residue 68. A novel *E. coli* transformant transformed with a recombinant vector in which the 5.5 kb SalI fragment of the *HpPIR2* gene of *Hansenula polymorpha* is inserted was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0827BP on July 11, 2000.

<1-3> Isolation of *HpGAS1* gene of *Hansenula polymorpha*

S. cerevisiae GAS1 gene was labeled by the Klenow fragment method with the aid of a DIG labeling system (Boehringer Mannheim) and used as a probe for southern blot analysis. Following hybridization at 42°C in a 30% formamide solution, signal was detected.

From the SalI fragment, a signal was detected at a size of about 1.6 kb. To identify the full length of the gene, the genome was treated with other restriction enzyme and hybridized with the same probe at 42°C in 20% formamide. As shown in Fig. 3, a signal was detected at a 3kb PstI fragment. This PstI fragment was cloned and inserted to pBluescript II

SK(+) at a PstI site to construct a recombinant vector, named HpGAS1. The DNA sequence analysis of the recombinant vector revealed that the HpGas1p of *Hansenula polymorpha*, encoded by the gene represented by the SEQ. ID. NO: 5, 1,614 bp long, consisted of 537 amino acids represented by the SEQ. ID. NO: 14, and shared homology of as high as 70.7% with the Gas1p of *S. cerevisiae*. The HpGas1p of *Hansenula polymorpha* was also found to possess a secretion signal sequence consisting of 18 amino acids, but GPI-anchor signal was different with that of S. cerevisiae.

A novel *E. coli* transformant harboring a recombinant vector in which the 3 kb PstI fragment containing *HpGAS1* gene of *Hansenula polymorpha* was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0828BP on July 11, 2000.

<1-4> Isolation of *HpTIP1* gene of *Hansenula polymorpha*

The open reading frame of the *Saccharomyces cerevisiae TIP1* gene was labeled according to the Klenow fragment method by a DIG labeling system (Boehringer Mannheim) and used as a probe for southern blot analysis for screening the *TIP1* homologue from *Hansenula polymorpha*. Following hybridization at 42°C

in a 30% formamide solution, southern blot signal was detected.

The southern blot analysis results are given in Fig. 4. At a 3.5 kb XbaI fragment, an apparent signal was detected. This 3.5 kb fragment was inserted to pBluescript II SK(+) at a XbaI site to construct a recombinant vector, named HpTIP1. The DNA sequence analysis of the recombinant vector revealed that the cloned gene had an 852 bp sequence represented by the SEQ. ID. NO: 6, coding for 283 amino acids represented by the SEQ. ID. NO: 13. A novel *E. coli* transformant harboring a recombinant vector containing the 3.5 kb XbaI fragment of the *HpTIP1* gene was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0824BP on July 11, 2000.

<1-5> Isolation of *HpCWP1* gene of *Hansenula polymorpha*

The open reading frame of the *Saccharomyces cerevisiae CWP1* gene was labeled according to the Klenow fragment method by a DIG labeling system (Boehringer Mannheim) and used as a probe for Southern blot analysis for screening the *CWP1* homologue from *Hansenula polymorpha*. Following hybridization at 42°C in a 30% formamide solution, southern blot signal was

detected.

As shown in Fig. 5, several weak signals were detected. Among the various signals, a 6kb SalI fragment showing the most clear signal was cloned into pBluescript II SK(+) at a SalI site to construct a recombinant vector, named HpCWP1. DNA sequence analysis disclosed that the cloned gene in the recombinant vector has a 246 bp sequences, which is expected to be an open reading frame represented by the SEQ. ID. NO: 7 with very low homology with the Cwp1p of *S. cerevisiae*. Additionally, the amino acid sequence, represented by the SEQ. ID. NO: 15, was expected to have a secretion signal consisting of 15 amino acids and a GPI-anchor signal predicted by the POSRT II prediction program. The corresponding protein was named HpCwp1p. It was expected to be a cell wall protein by its amino acids structure. A novel *E. coli* transformant harboring a recombinant vector containing the 6 kb SalI fragment coding for the HpCwp1p of *Hansenula polymorpha* was deposited with the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) under the accession No. KCTC 0826BP on July 11, 2000.

<1-6> Isolation of *HpWSC1* gene of *Hansenula polymorpha*

Based on the fact that fluorescence was observed

on the cell surface when a green fluorescence protein (GFP) was expressed as a fusion partner with Wsc1p, it was deduced that the amino terminal domain of Wsc1p could be used as a surface-expression mediator.

In the process of isolating the *LEU2* gene of *Hansenula polymorpha*, the *HpWSC1* gene was obtained by Agaphonov et al. (Agaphonov et al., *Yeast*, 1994, 10, 509, GenBank U00889). DNA sequencing analysis disclosed that the *HpWSC1* gene of *Hansenula polymorpha* consists of the 1,110 bp sequence represented by the SEQ. ID. NO: 8 encoding a 373 amino acid sequence. The protein encoded by the *HpWSC1* gene isolated from *Hansenula polymorpha* is a cell surface protein different in anchoring mechanism from GPI-anchor proteins and can be used to develop surface expression systems by its amino terminal domain.

EXAMPLE 2: Surface Expression System Using Cell Wall Proteins Isolated from *Hansenula polymorpha*

<2-1> Construction of surface expression system

Surface expression systems were constructed in which the four GPI-anchor protein genes and the *HpWSC1* gene, isolated from *Hansenula polymorpha* in Example 1, were used as mediator genes for surface expression of foreign proteins. Together with each mediator gene, a

CMCase gene derived from *Bacillus subtilis* (Park et al., *Agric. Biol. Chem.*, 1991, 55, 441) was used in the surface expression systems, functioning as a reporter gene.

In this regard, a GAPDH promoter (Sohn et al., *Appl. Microbiol. Biotechnol.*, 1999, 51, 800) and a killer toxin signal sequence (Sor, F. and Fukuhara, *Curr. Genet.*, 1985, 9, 147) were inserted into an AMIpL1 vector (Agaphonov et al., *Yeast*, 1999, 15, 541). Then, the CMCase gene was fused in frame to give a CMCase surface expression vector, named pGK CMCF, which could express CMCase under the control of the GAPDH promoter, as shown in Fig. 6. The CMCase gene stretch corresponding to an amino acid sequence from glycine at position 31 to aspartic acid at position 355 was obtained by PCR and linked downstream of the killer toxin signal sequence in the expression vector. As seen in Fig. 6, mediator genes were inserted into the carboxyl region of the CMCase gene between the recognition sites of restriction enzymes BamHI/HindIII. Also, the expression vector was designed to have an ARS, *HARS36* which directs the integration of vector to the telomeric regions of the chromosome of a host cell, so that comparison could be made of the translocation efficiency of surface anchor proteins (Sohn et al., *J. Bacteriol.*, 1996, 178, 4420) .

Four putative GPI-anchor protein genes, *HpCWP1*, *HpGAS1*, *HpTIP1* and *HpSED1* gene fragments, were amplified from the recombinant vectors by PCR using appropriate primer sets designed to provide BamHI and HindIII sites for the PCR products. The amplified putative GPI-anchor protein genes were inserted to the expression vector pGK CMCF at the BamHI/HindIII site located in the carboxy terminal region of the CMCase gene to construct CMCase surface expression vectors for *HpCWP1*, *HpGAS1*, *HpTIP1* and *HpSED1*, named CwpF, GasF, TipF and SedF, respectively.

Using primer sets containing BamHI/HindIII sites, four nucleic acid segments, each encoding the sequence of the 40 carboxy-terminal amino acids of each protein, were amplified by PCR, and inserted into the expression vector to construct CMCase surface expression vectors, named Cwp40, Gas40, Tip40 and Sed40, respectively.

For comparison of the surface anchoring efficiency, a DNA fragment, which encodes a carboxy-terminal sequence of 92 amino acids of Cwp2p, known as a GPI-anchor protein from *S. cerevisiae* excellent in anchoring efficiency, was synthesized by PCR and inserted into the same vector to construct a CMCase surface expression vector for Cwp2p, named ScCwp2, which was used as a control.

CMCase surface expression vectors containing the mediator protein genes were transformed into *Hansenula polymorpha* DL1-L (Hill et al., *Nucl. Acids Res.*, 1991, 19, 5791) and the transformants were selected in minimal synthetic media (2% glucose, 0.67% amino acid-free yeast nitrogen base, various amino acids with appropriate concentrations) devoid of leucine.

<2-2> Measurement of surface anchored CMCase activity

Each of the selected clones was inoculated in a YPD medium (2% glucose, 2% peptone, 1% yeast extract) and cultured at 37°C for 18 hours, after which the CMCase activity of the supernatant and the whole cell fraction were measured according to the DNS (dinitrosalicylic acid) method (G. L. Miller, *Anal, Biochem.*, 1959, 31, 426).

To test the CMCase activity, the culture broth was divided into a supernatant and a whole cell fraction by centrifugation. The whole cell fraction, after being washed twice with a citrate buffer (10 mM citrate buffer, pH 5.0), was suspended in the same buffer. The resulting suspension was added in a 1% CMC (carboxy methyl cellulose) solution and incubated at 55°C for 30 min. Reaction was stopped by boiling for 5 min in a DNS solution, followed by the measurement of absorbance at 550 nm. The results are given in Table 1,

below. The enzyme activity of CMCase was defined by U, and one U is the amount of enzyme activity which releases 1 μ mole of glucose from CMC for 1 min.

TABLE 1

Surface Expression Activity of CMCase According to anchoring Mediator

Anchoring Mediator	Activity in Supernatant (U/ml)	Activity in Whole Cell Fractio (U/ml)
Sed40	0.40	0.059
SedF	0.41	0.030
Tip40	0.92	0.090
TipF	0.45	0.024
Gas40	0.19	0.010
GasF	0.61	0.080
Cwp40	1.10	0.150
CwpF	0.65	0.110
MCMC	0.14	0.003
CMC*	1.92	0.000
ScCwp2	0.15	0.020
DL1	0.00	0.000

MCMC: Transformant harboring a CMCase expression vector with no secretion signal sequence.

CMC*: Transformant harboring a CMCase expression vector with a secretion signal sequence, a CMCase gene, and a stop codon instead of anchoring mediators

DL1: wild type cell with no expression vector.

In supernatants fraction, high CMCase activity was observed. This was believed to be attributed to

the fact that the CMCase expressed by the action of the GAPDH promoter was too much to properly anchored on the cell surface. Also, CMC, used as a substrate for CMCase activity assay, has the high molecular weight, so the enzyme anchored in the cell could not be effectively accessed to CMC. In whole cell fractions, the strains transformed with the vectors containing the mediator genes exhibit significantly improved CMCase activity, compared to those transformed with control vectors, MCMC devoid of the secretion signal sequence, and CMC° which had no mediator genes, thus demonstrating that CMCase was exported to the cell surface by each mediator. In addition, in transformant strains containing Tip40, GasF, Cwp40p and CwpF, CMCase were found to translocate to the cell surface at higher efficiency than those containing other mediators, as depicted in Fig. 7.

CMCase activity was also monitored with regard to culture time periods, and the results are graphed in Fig. 8. CMCase activity tended to decrease in most of the cells when they reached the stationary phase. But the CMCase activity of the cells, which harbored the surface expression vector having the *HpCWP1* gene, although being lowered in the stationary phase, was found to remain high throughout the culturing period

in the whole cell fraction. Therefore, HpCwp1p is inferred to be the most stable mediator among the proteins tested, as depicted in Fig. 8.

From the data obtained, HpCwp1p was identified to be superior in surface expression efficiency and stability among the novel four GPI-anchor proteins isolated from *Hansenula polymorpha*.

EXAMPLE 3: Surface Expression System Using HpPir2p

<3-1> Construction of surface expression system

A surface expression vector was constructed in which the *HpPIR2* gene encoding a cell wall expression protein different from GPI-anchor proteins was used as a mediator for surface expression of foreign proteins with CMCase as a reporter. To determine which part of the protein HpPir2p is useful for expressing target proteins on the cell wall, CMCase was fused to HpPir2p at its either termini, as shown in Fig. 9.

In order to utilize the carboxy terminus of HpPir2p for fusion with a target protein, a gene fragment encoding a polypeptide stretch ranging from the Kex2 cleavage site to the stop codon of the protein HpPir2p was synthesized by PCR and inserted to the expression vector, pGK CMCF constructed in Example 2, at the restriction enzyme sites (BamHI/HindIII) to give a recombinant expression vector, named Pir-C. In

the case of the amino terminus of HpPir2p, the expression vector, pGK CMCF was deprived of the killer toxin signal sequence and replaced with a gene fragment encoding the full length of the open reading frame from the initiator methionine to the stop codon of the HpPir2p, so as to fuse *HpPIR2* gene directly to the amino terminus of CMCase. The resulting surface expression vector was named Pir-N. The expression vectors were transformed into *Hansenula polymorpha*, followed by selecting transformants in the same manner as in above.

<3-2> Activity measurement of surface anchored CMCase

The CMCase activity of the transformants selected in Example 3-1 was measured, after being cultured in YPD media, and the results are given in Table 2, below.

TABLE 2

CMCase Activity of *Hansenula polymorpha* Using HpPir2p as Surface Expression Mediator

Mediator	Activity in Supernatant (U/ml)	Activity in Whole Cell Fraction (U/ml)
Pir-C #1	3.33	0.010
Pir-C #2	2.80	0.009
Pir-N #1	1.83	0.080
Pir-N #2	1.86	0.084
CMC*	2.53	0.000

As shown in Table 2, almost the same CMCase activity was measured when the carboxy-terminal region of the protein HpPir2p was used as mediator (Pir-C), as when a CMCase gene with a stop codon was used, indicating that the carboxy-terminal region of the protein HpPir2p was completely deficient in surface anchoring ability. Also, the CMCase activity was not affected by the insertion of the protein HpPir2P. By contrast, where the amino-terminal region of the protein HpPir2p was used as a mediator (Pir-N), CMCase activity was detected not only in the supernatant, but also in the whole cell fraction, demonstrating that the CMCase was expressed on the cell wall.

Therefore, the protein HpPir2p was anchored in the cell wall and thus could be used as a surface expression mediator. Over conventional GPI-anchor proteins, the protein HpPir2p has the advantage of linking the amino terminus of a target protein to its carboxy terminus. This anchoring protein is useful to avoid the problem of the activity loss took place when the target protein has an active site in its carboxy-terminal region.

EXAMPLE 4: Surface Expression System Using HpWsc1p

<4-1> Construction of surface expression system

The protein HpWsc1p is composed of a transmembrane domain by which the protein is anchored in the cell membrane, a serine/threonine-rich domain that traverses the cell wall, and an amino-terminal cystein motif which exists in the extracellular space, functioning to detect external signals (Verna et al., *Proc. Natl. Sci. USA*, 1997, 13804), as illustrated in Fig. 10a.

In this example, the *HpWSC1* gene was used for constructing a surface expression system. In this regard, CMCase was fused to the amino terminus of HpWsc1p and used as a reporter protein exposed on the cell surface.

To identify which domain is responsible for cell wall anchoring, truncated *HpWSC1* gene fragments were synthesized by PCR using primer sets designed to have BamHI/HindIII sites. Four truncated *HpWSC1* gene fragments, devoid of either the cystein motif (wsc-t) or the transmembrane domain (Wsc-c), and of both of them (Wsc-o), and an intact *WSC1* gene (Wsc-ct) encoding the full length of HpWSC1, were inserted at the BamHI/HindIII site of the plasmid vector (see Fig. 10b). The recombinant vectors were transformed into *Hansenula polymorpha* DL1-L, followed by selecting transformants in the same manner as in above.

<4-2> Activity measurement of surface anchored CMCase

After 18 hours culture in YPD broth, the CMCase activity of the selected transformants was measured according to the DNS method, and the results are given in Table 3, below.

TABLE 3

CMCase Activity of *Hansenula polymorpha* Strains Using HpWsc1p as Expression Mediator

Expression Mediator	Activity in Supernatant (U/ml)	Activity in Whole Cell Fraction (U/ml)
Wsc-c	0.90	0.002
Wsc-ct	0.35	0.069
Wsc-o	2.40	0.003
Wsc-t	0.04	0.006
CMC*	2.54	0.000
DL1	0.00	0.000

As seen in Table 3, only when the transmembrane domain was presented in the fusion protein (Wsc-ct, Wsc-t), the CMCase was not secreted, but rather remained attached to the cell. However, although the transmembrane domain was present, CMCase activity was not detected in the whole cell fraction if the cystein motif was absent (Wsc-o, Wsc-t). The result was believed to be attributed to the fact that the CMCase was not sufficiently exposed on the cell surface. In

contrast, when the transmembrane domain was absent (Wsc-o, Wsc-c), the CMCase activity was mostly detected in the supernatant. In addition, the cystein motif was found to make an additional contribution to the secretion of the CMCase.

Therefore, the mediator derived from the HpWsc1p must have both, the cystein motif and the transmembrane domain for anchoring efficiency. As the activity detected in the whole cell fraction was almost the same as in the case of GPI-anchor proteins, the HpWsc1p was suggested as a novel mediator for expression proteins on the cell surface.

EXAMPLE 5: Surface Expression of Glucose Oxidase

<5-1> Construction of surface expression system

Among the mediator proteins tested, three GPI-anchor proteins, HpTip1p (Tip40), HpGas1p (GasF) and HpCwp1p (CwpF), and the HpWsc1p (Wsc-ct) were tested for the surface expression of glucose oxidase. For the surface expression of glucose oxidase, a glucoamylase signal sequence and a glucose oxidase gene were synthesized by PCR and fused to each other before being cloned into pBluescript II SK(+). pGK CMCF vectors in which the expression mediator genes were included were treated with EcoRI and BamHI to remove the killer toxin signal sequence and the CMCase gene,

followed by the insertion of the glucose oxidase expression cassette to give a surface expression vector pGA GOD. This vector was transformed into *Hansenula polymorpha* DL1-L and transformants were selected in the same manner as in the above CMCase case.

<5-2> Activity assay of surface anchored glucose oxidase

The transformants selected in Example 5-1 were transferred onto YPD plate media and measured for glucose oxidase activity according to the plate activity assay method using peroxidase and O-dianisidine (Hodgkins et al., *Yeast*, 1993, 9, 625). As shown in Fig. 11, a smaller glucose oxidase activity circle was formed when expressing glucose oxidase with the anchoring mediators (Tip40, GasF, CwpF and Wsc-ct) than glucose oxidase without them (GOD*).

After being cultured in YPD media, the transformants, which were identified to have glucose oxidase activity on the cell surface by the plate activity assay method, were subjected to FACS analysis along with the wild type. The FACS results are given in Fig. 12. In this regard, the cultured cells were washed twice with phosphate buffered saline (PBS, pH 6.5) and suspended in PBS containing 1% BSA (bovine

serum albumin). The suspension was added with a glucose oxidase antibody (Accurate Chemicals) and allowed to stand for 1 hour on ice, after which the cells were washed three times with the same buffer and resuspended. A secondary antibody (FITC-labeled anti-rabbit antibody, Sigma) was added to the suspension which was incubated on ice for 30 min, followed by washing the cells three times with the same buffer.

In all cases of using the anchoring mediators, fluorescent cells were observed by FACS (fluorescence activated cell sorter) analysis, indicating that the glucose oxidase was expressed and anchored onto the cell surface. Additionally, higher glucose oxidase activity was detected in the whole cell fraction than in the supernatant throughout all of the strains. On the whole, the activity of surface anchored glucose oxidase was found to agree with that of CMCase. When CwpF was employed, fluorescence was detected from a large number of cells, demonstrating that CwpF had an excellent cell wall anchoring activity. It was also found that Tip40 was efficient in expression proteins in cell surfaces, as analyzed by FACS (Fig. 12). Under the mediation of GasF and Wsc-tc, fluorescence, as evidence of the cell wall anchoring, was detected, but in a low level, which resulted, to our knowledge, from the fact that GasF and Wsc-tc both are cytoplasmic

membrane proteins so that their exposure on the cell surface is poor compared to the other mediators. However, even in this case, there seems to be no problem in detecting the activity of the enzyme if the substrate penetrates the cell wall.

The assay results obtained from two enzymes anchored in the cell wall by use of various mediators, taken together, demonstrates that HpCwp1p is the most effective surface anchoring mediator among the GPI-anchor proteins and the proteins HpPir2p and HpWsc1p can be suggested as novel surface anchoring mediators.

EXAMPLE 6: Surface Expression System Using Glucoamylase Gene

<6-1> Construction of surface expression system

A known cell wall protein was investigated as to its usefulness as an expression mediator. The glucoamylase gene *STA1*, derived from *Saccharomyces diastaticus*, was obtained and studied for its usefulness to construct a surface expression system in *Saccharomyces cerevisiae*.

To begin with, various truncated *STA1* gene fragments were synthesized by PCR using appropriate primers and inserted to pBluescript II SK(+) at EcoRI and SmaI sites. The cloning was confirmed by DNA sequencing. The recombinant vectors were digested with

EcoRI and HindIII to create the site to which the GAPDH promoter of *Saccharomyces cerevisiae* could be inserted. After completion of the insertion, the digestion of the recombinant vectors with KpnI and EheI made various STA1 genes fused with the GAPDH promoter, which were inserted into the expression vector YEp352 (Hill et al., *Yeast*, 1986, 2, 163) at KpnI/PstI sites to construct CMCase surface expression vectors.

With reference to Fig. 13, there are shown vector diagrams for the surface expression systems using the *STA1* gene. As depicted, the surface expression systems were constructed with vectors comprising the signal sequence only (STASS), both of the signal sequence and the octapeptide sequence (STASS-8), all of the signal sequence, the octapeptide sequence and the threonine/serine-rich domain (STASS-8-TS), both of the signal sequence and the threonine/serine-rich domain (STASS-TS), both of the signal sequence and alpha-agglutinin (STASS-CMC-Agalc), and all of the signal sequence, the octapeptide, and alpha-agglutinin (STASS-8-CMC-Agalc), along with a CMCase gene as a reporter gene. The CMCase gene was synthesized by PCR, cut with SmaI/PstI and inserted into the expression vectors. The resulting expression vectors were introduced into *Saccharomyces cerevisiae* L3262, which

were then cultured in a minimal plate medium deficient in uracil to select the transformant, which harbored the expression vectors.

<6-2> Activity measurement of surface anchored CMCase

The transformants selected in Example 6-1 were assayed for CMCase activity after being cultured at 30°C for 48 hours in YPD media. The assay for CMCase activity was conducted by the pNPC (p-nitrophenyl β-D-cellobiocide) method (Deschpande et al., *Anal. Biochem.*, 1984, 138, 481). For this, the culture broth of each transformant was fractioned into a supernatant and a whole cell fraction by centrifugation. The cell mass was washed three times with PBS and suspended in PBS. The suspension was mixed with a 2.5 mM pNPC solution and incubated at 37°C for 1 hour, followed by adding an equal volume of 2% sodium carbonate (Na_2CO_3) to stop the reaction. Absorbance was measured at 410 nm, and the results are given in Table 4, below. In Table 4, the enzyme activity of CMCase was defined by U, one U being defined as the amount enzyme activity to releases 1 μ mole of pNP for 1 min.

TABLE 4

CMCase Activity in *Saccharomyces cerevisiae*

	ss-CMC	ss8-CMC	ssts-CMC	ss8ts-CMC
Activity in Supernatant (U/l)	339	389	189	145
Activity in Whole Cell Fraction (U/l)	13.6	14.5	112	128
Activity in Whole Cell Fraction Activity in Supernatant (%)	3.9	3.6	37	47

As shown in Table 4, when employing the signal sequence only (SS-CMC), most of the CMCase was secreted extracellularly, not anchored in the cell wall. In contrast, when employing the threonine/serine-rich domain linked directly to the signal sequence (SS-TS-CMC) or when employing the threonine/serine-rich domain linked to the signal sequence via the octapeptide sequence (SS-8-TS-CMC), the enzyme activity was measured to be low in the supernatant, but high in the whole cell fraction.

The same results were confirmed by plate activity assay, as shown in Fig. 14. Large circles were formed around the colonies, which harbored the signal sequence only (SS), as most of the expressed enzyme was extracellularly secreted. In contrast, the presence of the threonine/serine-rich domain hindered the enzyme secretion as proven by the small circles formed around the colonies.

To confirm the transport of CMCase to the cell surface, FACS analysis for CMCase was conducted in the same manner as in *Hansenula polymorpha*. The results are given in Fig. 15. As observed by the FACS diagrams,

fluorescence was detected in the cells harboring the threonine/serine-rich domain, suggesting the presence of CMCase on the cell surface. Meanwhile, after CMCase was fused to the carboxy terminus of α-agglutinin, CMCase activity was measured to test the surface anchoring ability. However, CMCase activity in this case was not higher than in case using only the threonine/serine-rich domain. Therefore, the threonine/serine-rich domain of the *STA1* gene alone was identified to function as a good surface anchoring mediator.

Thus, when used for the construction of surface expression systems, glucoamylase or its domains could be fused to the amino terminus of a target protein. Thus, these systems may circumvent the problems arising when a carboxy-terminal active domain of the target protein is interrupted by the mediator fused to the carboxy terminus. Therefore, the mediators described herein allow the development of surface expression systems capable of expressing various types of target proteins, according to the characteristics of the target proteins.

INDUSTRIAL APPLICABILITY

As described hereinbefore, surface expression

proteins derived from *Hansenula polymorpha*, an industrially useful methylotropic yeast, are highly effective in construction of surface expression systems and development of biocatalyst application systems. Because of their ability to produce industrially useful biomaterials, such as enzymes, antigens, antibodies, etc., and to offer industrial production tools such as immobilized biocatalysts, the surface expression systems established in the present invention can find numerous applications in various industries, including the medical industry, the food industry and the chemical and biochemical industry.

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above techniques. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : CHOI, Eui-Sung
Dasol Apt. 102-507, #395-3, Kung-dong, Yusong-ku, Taejon 305-335,
Republic of Korea/

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli DH5@/HpTIP1	KCTC 0824BP

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **July 11 2000.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	BAE, Kyung Sook, Director Date: **July 15 2000**

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Dusol Apt. 102-507, #395-3, Kung-dong, Yusong-ku, Taejon 305-335,
Republic of Korea/

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ***Escherichia coli*** **DH5@/HpSED1**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 0825BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

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TO : CHOI, Eui-Sung
Dasol Apt. 102-507, #395-3, Kung-dong, Yusong-ku, Taejon 305-335,
Republic of Korea/

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli **DH5@/IIpCWP1**	**KCTC 0826BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

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[ ] a proposed taxonomic designation
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INTERNATIONAL SEARCH REPORT

national application No.
PCT/KR00/00819

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
IPC7 C12N 15/52

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Electronic data base consulted during the internnational search (name of data base and, where practicable, search trerms used)
USPTO, NCBI pubmed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P	US 6,114,147 Sep 5, 2000 (05. 09. 00.)	1-10,11-14
A	US 5,223,408 June 29, 1993 (29. 06. 93)	1-10,11-14
A	US 5,223,409 June 29, 1993 (29. 06. 93)	1-10,11-14
A	US 5,602,034 Feb. 11. 1997 (11. 02. 97)	1-10,11-14
A	Mol Cell Biol 2000 Mar ;20(9):3245-55	1-10,11-14
A	Appl Environ Microbiol 1998 Dec;64(12):4857-61	1-10,11-14

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document publshed after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 JUNE 2001 (20.06.2001)	21 JUNE 2001 (21.06.2001)

Name and mailing address of the ISA/KR	Authorized officer
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